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Continuous Video Electroencephalogram during Hypoxia-Ischemia in Neonatal Mice

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TITLE:

Continuous Video Electroencephalogram during Hypoxia-Ischemia in Neonatal Mice

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KEYWORDS:

hypoxia; ischemia; electroencephalogram; neonate; encephalopathy; seizure

SUMMARY:

This manuscript describes a method for continuous video EEG recordings using multiple depth electrodes in neonatal mice undergoing hypoxia-ischemia.

ABSTRACT:

Hypoxia ischemia is the most common cause of neonatal seizures. Animal models are crucial for understanding the mechanisms and physiology underlying neonatal seizures and hypoxia ischemia. This manuscript describes a method for continuous video electroencephalogram (EEG) monitoring in neonatal mice to detect seizures and analyze EEG background during hypoxia ischemia. Use of video and EEG in conjunction allows description of seizure semiology and confirmation of seizures. This method also allows analysis of power spectrograms and EEG background pattern trends over the experimental time period. In this hypoxia ischemia model, the method allows EEG recording prior to injury to obtain a normative baseline and during injury and recovery. Total monitoring time is limited by the inability to separate pups from the mother for longer than four hours. Although, we have used a model of hypoxic-ischemic seizures in this manuscript, this method for neonatal video EEG monitoring could be applied to diverse disease and seizure models in rodents.

INTRODUCTION:

Hypoxic ischemic encephalopathy (HIE) is a condition that affects 1.5 in 1000 newborns annually and is the most common cause of neonatal seizures[1,2]. Infants who survive are at risk for various neurological disabilities such as cerebral palsy, intellectual disability, and epilepsy[3–5]. Animal models play a critical role in understanding and investigating the pathophysiology of hypoxia ischemia and neonatal seizures[6,7]. A modified Vannucci model is used to induce hypoxia ischemia (HI) on postnatal day 10 (p10)[7,8]. Mouse pups of this age translate neurologically roughly to the full term human neonate[9].

Continuous video electroencephalography (EEG) monitoring used in conjunction with this injury model allows for further understanding and characterization of neonatal hypoxic ischemic seizures. Previous studies have used various methods for analyzing neonatal seizures in rodents, including video recordings, limited EEG recordings and telemetry EEG recordings[10–16]. In the following manuscript, we discuss in depth the process of recording continuous video EEG in mouse pups during hypoxia-ischemia. This technique for continuous video EEG monitoring in neonatal mouse pups could be applied to a variety of disease and seizure models.

PROTOCOL:

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Virginia.

1. Electrode building/cable building

1.1. Use a unipolar insulated stainless-steel wire (0.005" bare diameter, 0.008" coated) to make an electrode that is connected with a female socket connector (female receptacle connector 0.079).

1.2. Use a special custom-made cable to connect animals to the amplifier.

1.2.1. Attach a male 4-pin connector (Male connector 0.079") to 4 channel unity gain impedance matching operational amplifier (op-amp). Attach a 10K resistor to the wires that connect to the 9 V battery. A ground wire not connected to the op-amp acts as the midpoint of the battery.

1.2.2. Connect one end of the cable (AWG, 0.012" OD) to the op-amp and connect the other end of the cable to the amplifier.

2. Electrode implantation surgery

2.1. Anesthetize the pup (postnatal day 9) with 4-5% isoflurane in a downward flow hood. Prior to the start of the procedure, inject the pups with bupivacine (0.02-0.05 mL, 0.25% local infiltration).

2.2. Once the animal is immobile, transfer to a stereotactic stage with a nose cone. Use the reverse side of the ear bar as it is soft to hold the head steady. At this age, pups do not have a

fully developed ear to use the pointed end of the ear bar.

2.3. Turn down the flow of isoflurane and maintain it at 2.5-3%. Keep an eye on steady breathing of the pup throughout the surgery procedure. Pinch the tail to check pain response and then proceed to incision.

2.4. Sterilize the incision area on the skull with betadine and alcohol (3 cycles of alternating iodine and 70% ethanol). Drape the surrounding body part such that the incision region is visible.

2.5. Open the scalp anterior-posterior from slightly above the eyes and retract approximately 1 cm of skin. Reposition the mouse head on the stereotaxic stage so that the skin pulls outward exposing skull.

2.6. Apply hydrogen peroxide on the skull using a cotton swab and scrape the skull clean using a scalpel blade. The skull is very soft; exercise caution while scraping.

2.7. Apply one drop (approximately 50 µL) of adhesive and spread it around the exposed skull area using its applicator. Expose to UV light for 40 s to set the adhesive.

2.8. Measure the coordinates using the exposed bregma as the reference. Implant electrodes bilaterally in the CA1 region of hippocampus [-3.5 mm Dorsal-Ventral (DV), ±2 mm Medial-Lateral (ML), -1.75 mm Deep (D)] and bilaterally in the parietal cortex [-1.22 mm DV, ±0.5 mm ML, -1 mm D] and a reference electrode in the cerebellum[17]. Use a 32 G needle to create a hole at the marked region.

2.9. Clean the blood from the surface of the skull. Lower electrodes attached to the female socket connector into the brain with the help of the stereotaxic arm and fix in place with dental acrylic. Implant the electrode in the brain. The socket connector headset sits on top of the skull glued together by dental acrylic.

2.10. Inject ketoprofen (5 mg/kg) subcutaneously once the electrode is fixed. Place the pups back with the mother.

NOTE: Introduce half of the litter with the headset at once to the mother rather than introducing them one at a time. This will prevent from mother damaging the pup's headset.

3. EEG setup and recording (baseline/pre-injury)

3.1. After 24 h of recovery after electrode implantation, place each animal in a heated (37 °C) custom-made Plexiglas chamber for EEG recording. This chamber will also serve as a hypoxia chamber.

3.2. Connect pups in the chamber to a video-EEG monitoring system via a flexible cable (custom made op-amp cable).

3.3. Digitize the EEG data at 1000 Hz with 1K gain using a grass amplifier. Review the EEG signal (band pass filter between 3-70 Hz) later using software (e.g., LabChart Pro).

3.4. Record a pre-injury baseline EEG for 30 minutes prior to disconnecting animals for carotid artery ligation procedure.

4. Left carotid artery ligation

4.1. Anesthetize the pup (postnatal day 10) with 4-5% isoflurane in a downward flow hood and place them on specially arranged setup on a waterbath pad. Position the animal supine and secure the forelimbs with paper tape.

4.1.1. Lower the flow of isoflurane to 2-3%. Pinch the tail for pain response and monitor breathing throughout procedure.

4.2. Sterilize the incision area (between mandible and the clavicle) on the left side of the neck with betadine and alcohol (3 cycles of alternating iodine and 70% ethanol).

4.3. Make an approximately 1 cm long incision on the left side of the neck using microscissors. Using a dissecting microscope, carefully retract the subcutaneous tissue and skin to expose the carotid artery. Take care to identify the vagus nerve (running lateral to the artery) and delicately separate and retract it from the artery.

4.4. Thread a 5 cm long sterile silk suture under the artery using microforceps. Tie a double knotted suture around the artery to occlude flow.

4.5. Cut the excess suture and close the exposed artery by pulling back the subcutaneous tissue and skin. Use vet bond to seal the incision.

4.6. Place the animal back on continuous EEG monitoring in a chamber at room temperature, which is placed on a warming mattress. Take spot infrared temperature checks of pup core temperature to avoid opening the chamber. Allow the animal to recover for 1 h prior to hypoxia.

5. EEG and hypoxia

5.1. Continuously monitor FiO_2 (fraction of inspired oxygen) within the chamber via an oxygen monitor.

5.2. Flush the chamber with 60 L/min of 100% N_2 and 0.415 L/min for 100% O_2. Once the oxygen saturation in the chamber reaches 12%, decrease the N2 flow to 10 L/min while keeping the O_2 flow unchanged. With small adjustments, maintain the FiO_2 at 8% for 45 min.

5.3. After 45 min of hypoxia exposure, return FiO_2 to 21%.

5.4. Have pups recover in chamber and monitor on EEG for 2 h post-hypoxia.

5.5. After completion of recording period, disconnect mice from EEG recording and return to the mother.

6. EEG analysis

6.1. Analyze the EEG file with video in LabChart Pro. Have a blinded researcher mark the EEG for seizures and background patterns[17]. Seizures are defined as an electrographic event lasting greater than 10 seconds with high frequency rhythmic sharp wave discharges (≥3x baseline) with clear evolution[17].

6.2. Have a second blinded researcher review marked events at random for agreement.

6.3. Review associated video for each marked electrographic event and analyze according to the neonatal rodent behavioral seizure score[16]. Briefly, this score ranges from 0-6 (immobility to severe tonic-clonic behavior). To further characterize seizure semiology, analyze behavior for laterality (multifocal/bilateral movements vs. focal/unilateral vs. mixed).

6.4. Create a power spectrogram. Use a Fast Fourier Transform with a Cosine-Bell data window with a size of 1024 data points. Create a smooth x-axis in the spectrogram with the help of a window overlap of 87.5%. Express the power as μV^2 [18].

REPRESENTATIVE RESULTS:

Seizure semiology

Neonatal hypoxia-ischemia exposure results in both generalized and focal seizures in mice (**Figure 1A-C**). Video EEG recordings allow electrographic findings to be correlated to behavior on video. These behaviors were scored using a previously published neonatal rodent behavioral seizure score (BSS)[16]. In addition to BSS, we categorized events based on whether the behavior was focal/unilateral, bilateral, or mixed (**Figure 1B**).

In this model, mice generally exhibited 3 patterns of seizure semiology: 1) repetitive circling to the side of ligation with extension of contralateral extremities, 2) loss of posture with body flexion and tail curled to side of ligation, or 3) loss of posture with unilateral or bilateral paddling of extremities (varying severity and length). The majority of observed events involved focal/unilateral or mixed behaviors (**Figure 1B**). In addition, during the hypoxic period, a subset of mice exhibited non-convulsive seizure activity, where the pup was immobile with sustained seizure activity on EEG (**Figure 1C**).

Electrographic recordings

EEG recording was started 30 minutes prior to carotid ligation in order to obtain a pre-injury baseline. Baseline activity (**Figure 1A** and **Figure 2A**) was similar to previously described background in p10 mouse pups[17]. Following ligation, pups were immediately placed back on

video EEG. During the period between ligation and commencement of hypoxia, a subset of mice exhibits convulsive seizures (**Figure 1A-C**).

Following hypoxia induction, background amplitude on EEG reduced (**Figure 3B**) and intermittently exhibited bursts of spike-wave discharges, followed by suppression (**Figure 2A**). Mice exhibit electrographic seizures, which emerge from a suppressed background as rhythmic spike-wave discharges and progress to become more complex and frequent, with polyspike waves (**Figure 2B**). During hypoxia, power spectrogram analysis was notable for asymmetries between the ischemic and contralateral hemisphere (**Figure 3A,B**). The ischemic hemisphere exhibited a burst suppression pattern and the contralateral hemisphere exhibited suppressed background (**Figure 1A** and **Figure 3A,B**). On average seizures begin 5.5±8.1 minutes after induction of hypoxia, with each event lasting 56±57 seconds. There was a 13% mortality rate during hypoxia (n=4/30), with all deaths following a convulsive (BSS=5-6) seizure.

During reoxygenation and recovery, a subset of mice continues to have seizures over the remainder of the recording period (2 h post-hypoxia). EEG background was suppressed compared to baseline following hypoxia (**Figure 1A** and **Figure 3**), with gradual recovery during the post-hypoxia recording period. Over the entire recording period, mice exhibited on average 9±5 seizure events, each lasted 54±57.7 s.

Figure Legends:

Figure 1: Seizure characteristics in p10 mice exposed to neonatal hypoxia–ischemia. (**A**) Representative power spectrogram from the ischemic parietal cortex electrode through the experimental timeline. (Amplitude color heat map scale $x10^{-6}$). Arrows indicate the time that raw electroencephalogram tracings below the spectrogram represent. (**B**) Seizure behaviors for the entire experiment, postischemia/prehypoxia, during hypoxia, and posthypoxia. (**C**) Behavioral seizure score (BSS) and timing for all seizure events (n = 30 mice, each mouse has a unique symbol, each point is a discrete seizure event). 100% of mice seized during hypoxia (blue box; time = −60 minutes is the completion of carotid ligation, time=0 is the start of hypoxia). Thirteen percent died during hypoxia following a convulsive seizure (grade 5–6). This figure has been modified from Burnsed et al[13].

Figure 2: Characteristic electroencephalography (EEG) patterns during hypoxia ischemia. (**A**) EEG background from left to right: preinjury baseline, burst suppression during hypoxia, posthypoxia suppression. Recording from ipsilateral parietal cortex depth electrode. (**B**) Evolution of a seizure during hypoxia. Recording from ipsilateral hippocampal depth electrode. Shaded boxes (I-V) corresponded to expanded EEG excerpts on the right of (**B**). This figure has been modified from Burnsed et al[13].

Figure 3: Asymmetries in EEG background between ischemic and contralateral hemispheres. (**A**) Asymmetric power spectrogram in HI mice during hypoxia (45-minute period) in ischemic cortex (left) and contralateral cortex (right; amplitude scale $x10^{-6}$). Burst suppression pattern and seizures in ischemic hemisphere, suppression in CL hemisphere. (**B**) Background suppression during hypoxia and reoxygenation in IL and CL hemispheres. All measurements of

mean voltage taken from 10-second random excerpts of the encephalogram over the experimental time period (baseline, 30 minutes postligation, during hypoxia—15 minutes and 30 minutes after start, after reoxygenation—15 minutes and 60 minutes after start) were compared to baseline. Each animal's baseline served as its own control, and data are reported as a percentage of baseline (n = 5 mice). Measurements were taken from cortical electrodes. This figure has been modified from Burnsed et al[13].

DISCUSSION:

We have presented a model for continuous video-EEG monitoring in neonatal mice during hypoxic-ischemic seizures. Video analysis in conjunction with EEG allows characterization of seizure semiology. Analysis of EEG allows for extraction of power spectrograms and background amplitude analysis.

Correct and careful placement of electrodes is crucial in this protocol, as injury during electrode placement or inaccurate placement can significantly affect results. Assessment of normal baseline EEG activity prior to injury is paramount, as bleeding or injury during electrode placement, while rare, can happen. Secondly, in order to confirm correct electrode placement, brains can be sectioned and examined for electrode tracks in the proper placement. In addition, failure to return pups to the mother in groups (individually) may result in electrode headsets being damaged or pups being killed or neglected by the mother.

One limitation of this method is the limit of spatial localization of depth electrode recordings in a small neonatal brain. This restricts the ability to localize specific seizure foci on EEG recordings. Another limitation in this model of hypoxia ischemia is the variability in seizure burden. Variability in lesion size and behavioral deficits in this rodent model of hypoxia ischemia has been well described previously[7,8,19]. Not surprisingly, this variability exists in seizure burden (both length of seizure events and number of seizure events). However, consistently, 100% of pups in this model exhibit seizures during hypoxia. Lastly, the amount of time pups can be on EEG monitoring (away from the mother) is limited. Therefore, we are unable to characterize ongoing seizures with continuous EEG at later time points relative to the injury.

Although, we have used a hypoxia-ischemia seizure model in this manuscript, this method for continuous video-EEG monitoring in neonatal mouse pups could be easily applied to other disease/seizure models. Seizures in neonatal rodents are difficult to recognize based on behavior alone, making video-EEG monitoring important. Future investigations could use these techniques to analysis seizure burden and semiology in other neonatal seizure models or response to therapeutics and neuroprotective measures.

ACKNOWLEDGEMENTS:

We acknowledge the following funding sources: NIH NINDS – K08NS101122 (JB), R01NS040337 (JK), R01NS044370 (JK), University of Virginia School of Medicine (JB).

DISCLOSURES:

None

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352 unique microstructural information in normal and hypoxia-ischemia injured mouse brains.
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354

Figure 1

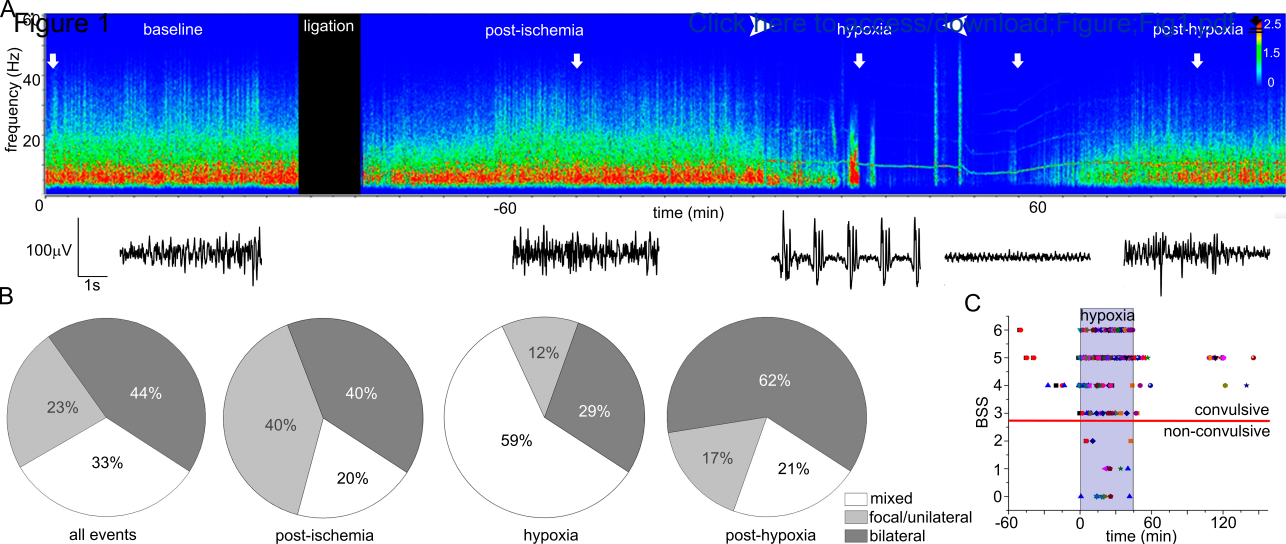
A
baseline
ligation
post-ischemia
hypoxia
post-hypoxia
frequency (Hz)
40
20
0
2.5
1.5
0
-60
time (min)
60
100µV
1s
B
44%
23%
33%
all events
40%
40%
20%
post-ischemia
12%
29%
59%
hypoxia
62%
17%
21%
post-hypoxia
mixed
focal/unilateral
bilateral
C
hypoxia
BSS
convulsive
non-convulsive
time (min)

Figure 2

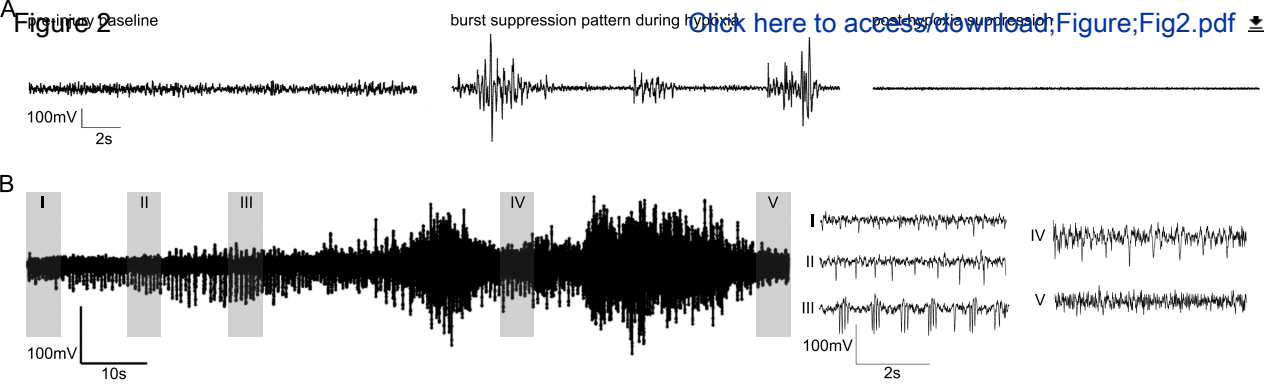

Figure 3

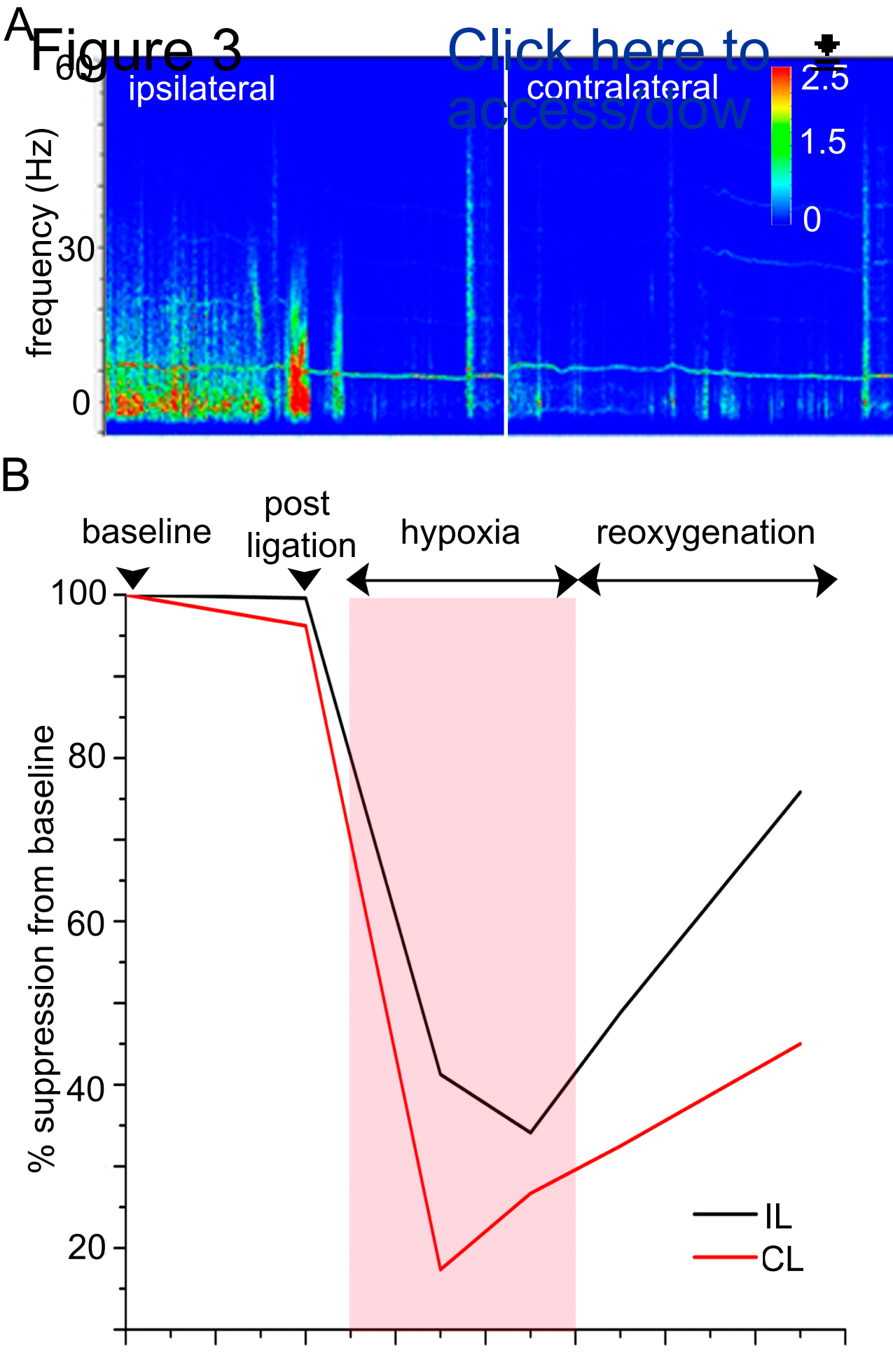

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
SURGERY		
Ball Point Applicator	Metrex Research	8300-F
Cranioplast (Powder/Resin)	Coltene	H00383
I-Bond	Kulzer GmbH, Germany	
LOOK Silk Suture	Surgical Specialities Corporation	SP115
RS-5168 Botvin Forceps	Roboz Surgical Instrument	RS5168
RS-5138 Graefe Forceps	Roboz Surgical Instrument	RS5138
UV light for I-Bond	Blast Lite By First Media	BL778
Vannas Microdissecting Scissor	Roboz Surgical Instrument	RS5618
Vet Bond	3M Vetbond	1469SB
HYPOXIA		
Hypoxidial	Starr Life Science	
Oxygen sensor	Medical Products	
EEG RECORDING		
Female receptacle connector 0.079"	Mill-Max Manufacturing Corp	832-10-024-10-001000
Grass Amplifier	Natus Neurology Incorporated	
LabChart Pro	ADI Instruments	
Male Socket Connector 0.079"	Mill-Max Manufacturing Corp	833-43-024-20-001000
Operational Amplifier	Texas Instruments, Dallas, TX, USA	TLC2274CD
Operational Amplifier	Texas Instruments, Dallas, TX, USA	TLC2272ACDR
Stainless Steel wire	A-M Systems	791400
Ultra-Flexible Wire	McMaster-Carr	9564T1

Comments/Description

i-bond applicator
Perm Reline/Power

LOOK SP115 Black Braided Silk Non absorbable surgical suture
Forcep for surgery/ligation
Forcep for surgery/ligation
UV ligth for I-bond
Scissor for ligation
Vet Glue

MiniOxI- oxygen analyzer/sensor for hypoxia rig

Ordered from Digikey
Grass Product
Software to run the system
Ordered from Digikey
TLC2274 Quad Low-Noise Rail-to Rail Operational Amplifier
TLC2274 Quad Low-Noise Rail-to Rail Operational Amplifier
0.005" Bare/0.008" Coated 100 ft
36 Gauze wire of various color

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Linda Crawford
Steve Garrison
Tammy Mawyer

Research Coordinators
Monika Thielen
Joey Michel

May 5, 2020

Dear Dr. DSouza,

Please find enclosed revisions on our manuscript entitled "Continuous Video Electroencephalogram during Hypoxia-Ischemia in Neonatal Mice" (JoVE61346). We thank the reviewers and editors for their time and thoughtful review.

Please see our response to reviewers letter below and the attached revisions in the manuscript (with tracked changes in MS Word document).

This manuscript is being submitted only to *Journal of Visualized Experiments* and will not be submitted elsewhere while under consideration. Further, it has not been published elsewhere in whole or in part.

All authors are responsible for reported research and have participated in the concept and design, analysis and interpretation of data, and/or drafting or revising of the manuscript. They have approved the manuscript as submitted. We have no conflicts of interest other than NIH funding to Dr. Kapur and myself.

Sincerely,

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Response to Reviewers

Reviewer #1:
Manuscript Summary:
The authors of "Continuous Video Electroencephalogram during Hypoxia-Ischemia in Neonatal Mice" offer a detailed protocol for implantation of an electrode into the brain (hippocampus, parietal cortex) to measure seizure EEG in mouse pups. The implantation surgery is described, and conducted 24 hours prior to carotid artery ligation and hypoxia to produce brain injury. This injury procedure is based on the common and accepted Rice Vannucci model. Pups are subjected to HI at postnatal day 10. This is considered a model of term birth. The demonstrated that EEG can be measured continuously during hypoxia, and for at least 2 hours post hypoxia. The nature of the seizures tends to change after hypoxia, from largely constrained to the ipsilateral hemisphere to both hemispheres. The authors also describe the behavioral indicators of seizures and note that the EEG can be matched to behavior using their dual video/EEG data acquisition set up. The authors carefully list vendor catalog numbers and various details to promote success of others that might chose to use this technique. Overall, the methods are clear, and since this technique is new to this reviewer, but the HI procedure is not, I assume that the EEG protocol is as detailed at the HI protocol. Indeed, all critical details are present in the HI protocol. The authors make the important note that the pups should be returned to the dam in a group after electrode implantation. I am eager to see this published in video format.

Major Concerns:
No major concerns

Minor Concerns:
No minor concerns

Reviewer #2:
Manuscript Summary:
The authors describe a method for monitoring video/EEG from neonatal mouse pups immediately before, during and immediately after hypoxia/ischemia. They provide a clear description of the method which is placed into context with regard to neonatal stroke. The method provides a potential means to understand neuropathological mechanisms associated with neonatal hypoxia/ischemia. There are a couple of concerns with the manuscript:

Major Concerns:
1) It's useful to have baseline EEG before hypoxia as a control as indicated. However, it would be helpful to also see results from sham operated controls to define the impact of electrode placement and carotid surgery without ligation.
For each mouse we have performed a minimum of 30 minutes of baseline (pre-injury) EEG recording so that each mouse serves as its own control. This allows us to control for any impact of electrode placement. Recordings noted to have excessive artifact or other issues with recording are excluded. In addition, gross brains are examined following the experiment to verify correct electrode placement.

2) It's not clear if EEG recording is conducted before ligation of the carotid artery. This should be clarified.
Baseline recording is conducted for 30 minutes prior to ligation of the carotid artery. Please see lines 126-127 of the protocol.

3) Is occlusion of one carotid adequate to induce ischemia? Do pups have an underdeveloped circle of Willis? Does the contralateral carotid not provide adequate collateral flow?
Yes, this is adequate to induce ischemia. This is a well-established model of neonatal hypoxic ischemic injury which has been used in this field for over 30 years and results in a significant ischemic injury which has been thoroughly described in numerous studies (Rice 1981, Burnsed 2015, Ferriero 2019).

Minor Concerns:
1) in section 4.3 please indicate where the incision is being made.

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This has been clarified, please see line 135.

Reviewer #3:

Manuscript Summary:

The manuscript described methodology of performing neonatal hypoxia-ischemia treatment in mouse pups. The authors include surgical techniques, electrode implantation as well as examples of analyzed data. For a reader to be able to reproduce methodology described in this paper, more details would be necessary around how electrodes are placed in the animal. Methodology about data acquisition and analysis is insufficiently described in the manuscript.

Major Concerns:

Data acquisition parameters should be included, as well as how data was processed to generate figures. Additionally, several methods such as BSS scoring are cited, but not described directly in the manuscript. In my opinion, it would be useful for the reader to include the details of these techniques directly in this manuscript. Finally, it would be useful to include a very specific and detailed example of what the authors considered to be a seizure. Was there a difference in EEG between convulsive and non-convulsive events?

Thank you for these comments. As you recommended, we have now included more specifics in the protocol regarding protocol to generate power spectrogram figures and BSS, reference lines 310-311, 314-317. We have also included more details on the definition of seizure that we cited in the protocol, lines 305-307.

Specifics:

Figure 1: It is not clear what authors consider to be "seizure" activity. Panel A shows raw traces of what appears to be background activity, isoelectric EEG and some spiking activity. How is seizure defined? Is it possible to show a trace of what is considered to be a "seizure"? How long are events that are considered to be seizures? It would also be useful to include longer raw EEG traces in addition to spectrogram. Generation of spectrogram is not described in the methods.

We have now defined the criteria we used for seizures in this study (based on previous citations) in the manuscript, lines 305-307. Figure 2 shows a trace of a seizure, with time scale to indicate length of this example seizure. We have also included in the revised manuscript the protocol used to generate the spectrogram, lines 314-317.

Figure 2: The event in B is difficult to interprete. Consider temporally expanding the trace, so that activity is not merged together.

Because of the length of the event used in this example, an expanded trace for the entire event would be cumbersome in a figure. We have demonstrated key patterns on expanded EEG to the right of the trace. Each of these expanded sections represents the correlative region of EEG in the shaded box on the left. The figure legend for this figure has been modified to improve clarity (line 453).

Figure 3: How is data analyzed? Is it percent or raw power? RMS power? Power in certain frequency bands? More details are necessary for reader to be able to replicate this analysis.

We have included more details in the protocol regarding the analysis of EEG data, lines 314-317. Figure 3B is total power as a percentage of each animal's baseline, see figure 3 legend for these details. Frequency range used is 0-60Hz.

Minor Concerns:

Line 80: What is iBond? Why use it before electrode implantation

iBond is an adhesive which helps fix the electrodes in place. We have added this to the revised protocol, line 92.

Line 88: What happens to electrode after it is placed with acrylic? Is it routed outside the animal somehow? How is it protected from the mother? Does the electrode have some kind of connector? More details in this part would be useful.

The electrode in fixed in place with a low profile headset device. The electrode extensions are connected via flexible cable at the time of EEG recording. We have included these details in the protocol, lines 99-102, 122-123.

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Line 88: Consider using "lowered into the brain" or "inserted into the brain" instead of "dropped into the brain"
Thank you, we made this modification, line 100.

Line 95: What temperature are pups maintained at? Is humidity maintained?
Intermittent body temperature checks are performed using an infrared detector to ensure consistent temperatures between 36-37.5℃. The surgical field and hypoxia/recording chamber are on circulating water warming mats. Humidity is the same as the ambient air, not continuously monitored.

Line 98: What are the data acquisition parameters? Sampling rate? Instructions about how to make the "custom op-amp" cables would be useful for reproducibility
We have included additional detail on the data acquisition parameters, please see lines 124-125. We digitize the data at 1000Hz with a 1K gain using the grass amplifier. EEG signal is reviewed using LabChartPro (v8) using a band pass filter between 3 and 70Hz. Details on custom op-amp cables are now included on lines 62-72.

Line 106: What are the gain and other settings on the amplifier. More details would be useful
We have included additional details in the protocol, lines 124-125. We digitize the data at 1000Hz with a 1K gain using the grass amplifier.

Line 108: Consider using "Disconnecting" instead of "unhooking"
Thank you, we made this modification, line 126.

Line 119: Which carotid is being ligated? Right or Left? How is the vagus nerve separated from the artery? Is 1 knot enough to completely occlude the blood flow? With one knot, is there reperfusion in the days following the procedure?
The left carotid is ligated, see line 135. A dissecting microscope is used to visualize the anatomy. The vagus nerve is identified, gently separated from the artery using blunt dissection, lines 137-139. Ligation is accomplished using a double knot, as previously published by several groups (Rice 1981, Burnsed 2015, Ferriero 2019). While reperfusion may occur days to weeks after the procedure, this does not affect the results of this experiment, which examines only acute seizures in this injury.

Line 126: How long is recovery time between carotid ligation and hypoxia?
Recovery time is 1 hour, this is included in the protocol, see line 146.

Line 128: Define FiO2 abbreviation
Thank you, we made this modification, line 148.

Line 135: Consider using "Disconnected" instead of "unhooked"
Thank you, we made this modification, line 301 .

Line 166: Consider using "amplitude was reduced" instead of "amplitude on EEG declined"
Thank you, we made this modification, line 342.

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